

The Effects of Baking on the Action of Trypsin Inhibitors in Soy Bread

Thesis

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1-Introduction

1.1 Role of Trypsin in the Body

Trypsin, a serine protease, is produced in the pancreas as the zymogen trypsinogen, and stored in its inactive form so as not to kill pancreatic cells by cleaving cytoplasmic proteins (Horton and others 2006). After a meal, the pancreas is stimulated by cholecystokinin and the zymogen is sent to the small intestine where it is activated by specific proteolysis by the enzyme enteropeptidase, cleaving the lysine-6 – isoleucine-7 bond in the protease. Once this takes place, the trypsin is free to activate other serine proteases, including chymotrypsin, elastase, and more trypsin (by autocatalysis)(Horton and others 2006). Once this step is completed, trypsin cleaves peptide bonds containing carbonyl groups from arginine and lysine (Horton and others 2006).

1.2 Action of Soy Trypsin Inhibitors

Two types of protease inhibitors are found in soy: the Kunitz trypsin inhibitor (KTI) and the Bowman-Birk inhibitor (BBI). KTI is a strong inhibitor of trypsin, and is significantly larger than BBI at 20,100 daltons. BBI inhibits both trypsin and chymotrypsin and is roughly 8,000 daltons (DiPietro and Liener 1989). When inhibitors are in the presence of trypsin, the active site of the enzyme is blocked, an irreversible compound is formed instantly, and trypsin is rendered ineffective (Blow and others 1974; Kunitz 1947). If ingested proteins are unable to be hydrolyzed and broken down into smaller pieces by trypsin (and the other proteases it can activate) due to trypsin inhibitors, it can result in gastric distress and lead to pancreatic hypertrophy or hyperplasia (Horton and others 2006; Smith and others 1989). In rodent models, animals fed either soy protein concentrate or direct concentrate of trypsin inhibitor

showed a dose-related increase in pancreas weight due to both hyperplasia and hypertrophy (Smith and others 1989). This conclusion indicates that long-term consumption of a diet high in soy products with strong trypsin inhibitor activity may produce the same undesirable effects in humans, as well.

1.3 Value of Soy and Quantification of Trypsin Inhibitors in Soy Ingredients

Food scientists at The Ohio State University have explored the incorporation of soy flour and soy milk powder in baked products to confer certain health benefits. Specifically, soy bread and soy-almond bread have been used as an adjuvant therapy to men with prostate cancer. However, trypsin inhibitors may diminish the value of soy in these novel food systems. Smith and others (1989) discovered that the amount of soy inhibitors is directly related to the level of inhibition seen in the body. Therefore, a product with two sources of soy such as the bread developed at OSU is suspect to produce inhibition of trypsin in the body. Yet, there is a modicum of research that outlines the levels of trypsin inhibitors in common soy products. Trypsin inhibitors can be inactivated by heat, and since soy is typically processed to enhance its nutritional value, some dismiss the inhibitors as an issue (Liener 1986; Venter 1999). However, the heat sufficient to destroy trypsin inhibitors may be too high to preserve the nutritional quality of soy. Thus, a balance must be in place during processing. Based on this, it is estimated that 5-20% of trypsin inhibitors from the original soybeans remain active in commercially available soy products (Liener 1986).

Given that soy flour and soy milk powder are heat treated, it can be speculated that a significant proportion of the original level of inhibitors is decreased in the processing of these ingredients. Moreover, since both products are baked, it can be expected to see an additional

decrease in trypsin inhibitor levels. Therefore, the objective of this research was to determine if soy bread contains active trypsin inhibitors after the baking process. Further, if the bread exhibits trypsin inhibitor activity, to identify the ingredients that yield trypsin inhibition in soy bread, and to quantify the levels of inhibition in each.

2-Materials and Methods

The following procedure is based on AACC method 22-40 (2010).

2.1 Protein extraction of soy ingredients

The soy products evaluated in this research were soy flour (Baker's soy, ADM Protein Specialties Division; Decatur, IL), soy milk powder (Devansoy; Carroll, IA), and soy bread (developed at OSU). The bread was ground in a blender (KitchenAid) to at least 100 mesh, without the generation of heat. Each product was extracted in a ratio of 1 g ingredient to 50 mL 0.01 NaOH for three hours, with a magnetic stirrer on low setting.

2.2 Determination of Trypsin Inhibitor Activity

Reagents:

1. Tris buffer: 6.05 g tris (hydroxymethylamino) methane and 2.94 g $\text{CaCl}_2 \cdot \text{H}_2\text{O}$ in 900 mL water.

Adjust pH to 8.2, dilute volume to 1 L with water.

2. Substrate solution: 40 mg benzoyl-DL-arginine-*p*-nitroanilide hydrochloride (BAPA) (Sigma Aldrich) in 100 mL Tris buffer.

3. Trypsin solution: 4 mg trypsin (Sigma Aldrich) in 200 mL 0.001M HCl.

4. Acetic acid solution: 30 mL glacial acetic acid in 70 mL water.

Portions of the extract were pipetted into duplicate test tubes (0, 0.6, 1.0, 1.4, and 1.8 mL) and adjusted to 2.0 mL with water. Each test tube received 2.0 mL of trypsin solution and was agitated and heated in a water bath to 37 °C. Into each tube, 5.0 mL of BAPA solution (previously heated to 37 °C) was added, and all tubes were replaced in the agitating water bath. After exactly ten minutes, the reaction was stopped with 1.0 mL of acetic acid solution. Each solution was filtered through Whatman number 2 syringes, and measured at 410 nm on a spectrophotometer (Shimadzu). The blank was prepared by mixing 2.0 mL of sample extract and 5.0 mL of BAPA, heating at 37 °C for ten minutes, adding 1.0 mL of acetic acid solution, followed by the addition of 2.0 mL of the trypsin solution. Pure trypsin inhibitor (Sigma Aldrich) was also tested in place of soy products as a control.

2.3 Calculation of Trypsin Inhibitor Activity

According to the AACC method, an increase of 0.01 in absorbance at 410 nm per 10 mL of reaction mixture is equivalent to one trypsin unit (2010). Thus, one trypsin inhibitor unit, or TIU, can be defined as a decrease in absorbance by 0.01. The absorbances for each tube were averaged and divided by 0.01, then the differences between each tube were calculated successively to acquire TIU/mL of suspension. TIU/mL of suspension was then plotted against volume of extract and extrapolated to zero. If this graph is linear, then TIU/g can be calculated by multiplying the extrapolated value by the dilution factor of the respective tube. If the graph of TIU/mL of suspension versus volume of extract does not have a linear correlation, TIU/g should be calculated using the average values for TIU/mL multiplied by the dilution factor.

2.4 Optimization of Assay

2.4.1 pH Monitoring

The pH of the suspension should be maintained between 8.4 and 10.0 to optimize the action of trypsin on the substrate, BAPA. If not kept within this interval, the absorbances reflect the low activity and typically are not in acceptable ranges for data collection.

2.4.2 Concentration of enzyme and substrate

In order to achieve absorbances within an acceptable spectrophotometric range (0.1-1.0), the concentration of trypsin and BAPA can be tripled. The assay is most effective when the concentrations of both solutions are increased, rather than just one.

2.4.3 Elimination of Fat

Free fatty acids in soy products can act as strong inhibitors of trypsin, thus interfering with the basic nature of this assay (Smith and others 1980). Three different methods were used to eliminate fat from the soy product before extraction. The first method mixed 50 mL of cold hexane with the sample in a separatory funnel for one minute, and the fat layer was removed. This process was completed three times for each sample. The second method was soxhlet extraction, which started with 5 g of each soy product added to individual cellulose extraction thimbles. Each thimble was placed inside the soxhlet extraction chamber, and round bottom receiving flasks with 125 mL of petroleum ether and boiling beads in each were attached to the bottom of the chambers. The heat was turned on to medium and the samples were refluxed for twelve hours. After the extraction was complete, the soy products were removed for protein extraction for the assay. Third, centrifugation of the samples was performed at 10,000 x g for

15 minutes at 4 °C (Sorvall RC5C Plus with SM-24 Rotor; Thermo Scientific; Waltham, Massachusetts).

3-Results and Discussion

3.1 Quantification of Trypsin Inhibitors

Figure 3.1 represents the relationship between trypsin activity with increase of soy sample extract. If uninhibited, trypsin cleaves BAPA to product p-nitroaniline, a yellow pigment that absorbs at 410 nm. If trypsin is inhibited, the absorbance will be low. When pure trypsin inhibitor was tested, as volume increased, the inhibitory action increased as well, and trypsin was unable to cleave BAPA, indicated by the decrease in absorption. Soy bread shows the best linear relationship between these two factors, suggesting that it contains trypsin inhibitors. However, soy flour has an average absorbance of 0.25. Therefore, the trypsin in the reaction was able to act without interference, indicating that this soy ingredient contains no trypsin inhibitors.

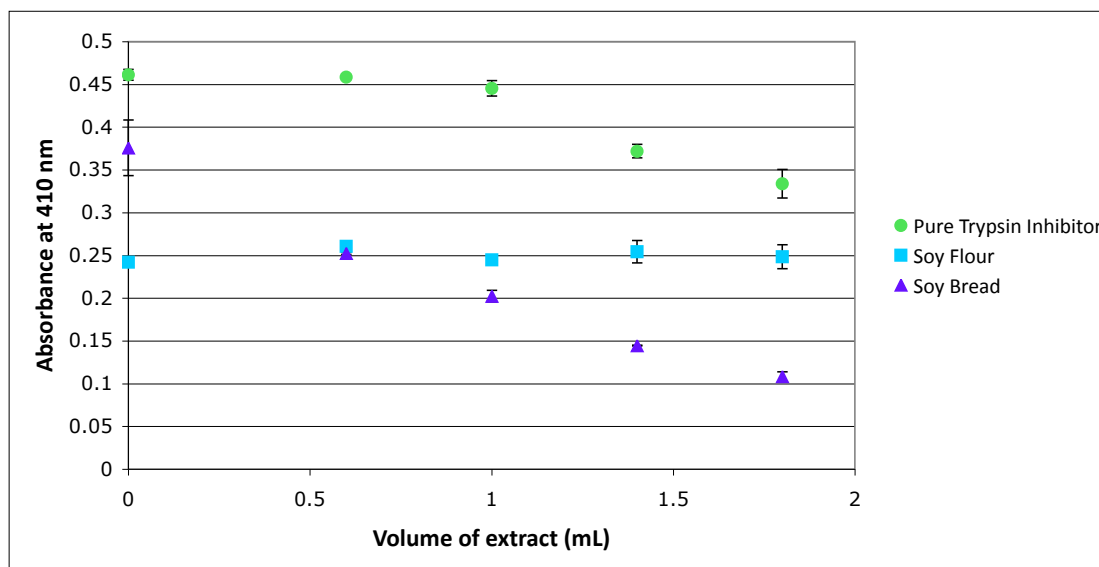


Figure 3.1. Absorbance at 410 nm as it relates to volume of extract and trypsin activity for pure trypsin inhibitor, soy flour, and soy bread.

Figure 3.2 shows the actual values of trypsin inhibitor units per mL of suspension and the relation to the volume of the extract, or amount of soy sample. In this graph, slope is indicative of the amount of trypsin inhibitors contained within each sample, as the final extrapolated value is that which gives the TIU/g of soy ingredient. Soy flour has a slope of zero, which supports the evidence in Figure 3.1 that there are no trypsin inhibitors in this soy ingredient. There is a strong, positive linear correlation between TIU/mL and volume of extract for pure trypsin inhibitor, which is to be expected. For soy bread, there is also a positive linear correlation, further indication that trypsin inhibitors are present.

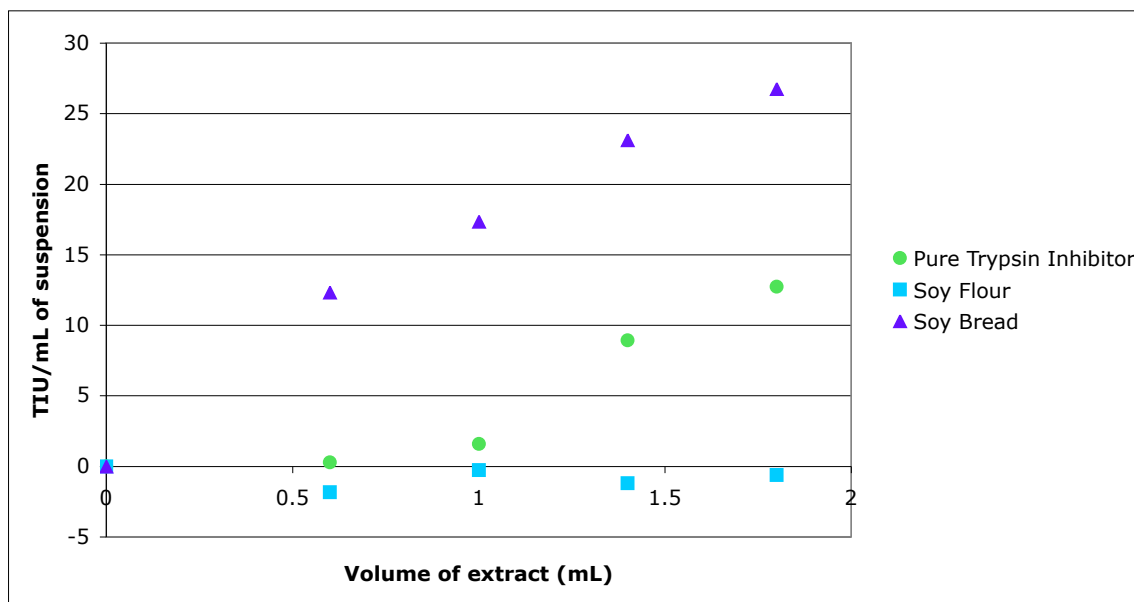


Figure 3.2. Calculation of TIU/mL of suspension of pure trypsin inhibitor, soy flour, and soy bread.

The results from final calculations of TIU/g of soy product are shown in Table 3.1. As expected, the soy flour has no trypsin inhibitor units per gram of flour. This indicates that the heat treatment undergone by the flour is sufficient to eliminate 100% of inhibitory activity. Soy

bread, however, contains 2.89 TIU/g of bread. This was not expected, as it was hypothesized that the baking process would inactivate any residual trypsin inhibitors the in the soy ingredients and result in no inhibitory activity. Throughout the testing, soy milk presented a number of issues with the assay, which led to the extensive optimization outlined above. Since inconclusive results were found for this soy ingredient, it can be hypothesized that soy milk powder is the ingredient in soy bread that contributes trypsin inhibitors.

Table 3.1. Trypsin inhibitor units per gram of soy flour and soy bread.

Sample	TIU/g
Soy Flour	0.00
Soy Bread	2.89

4-Conclusions

From the results, it can be concluded that soy flour is a viable option for incorporation in other soy-based foods without the consequence of trypsin inhibition in the body. While soy bread contains 2.89 TIU/g of bread, this should not be cause for alarm. This is a very low level of trypsin inhibitors, and will not cause significant distress to the gastric system of the consumer. Since soy flour exhibited no inhibitory activity, it can be inferred that soy milk powder is the cause of trypsin inhibitors in the soy bread. However, optimization of the assay did not prove to be entirely successful for this ingredient. Thus, further testing and optimization must be performed to quantify the levels of trypsin inhibitors in this ingredient. Once definitive results are determined, the reduction of trypsin inhibitors by baking can also be assessed.

Final Note: The author of this thesis and research also wrote the Wikipedia page on trypsin inhibitors.

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